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Detection of Hepatitis E Virus RNA in Stools and Serum by Reverse Transcription-PCR

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Stools and sera collected during an experimental hepatitis E virus (HEV) infection in monkeys and collected from humans with acute HEV infections during epidemic and sporadic cases were analyzed by reverse transcription-PCR. Two methods for RNA purification were compared. Proteinase K digestion and phenol-chloroform extraction were more efficient than guanidinium isothiocyanate extraction in improving the sensitivity and specificity for the detection of HEV genomes.

Hepatitis E virus (HEV) has been reported in both epidemic and sporadic cases of hepatitis in developing countries (3). Several species of nonhuman primates have also been experimentally infected with HEV (4). Molecular methods provide tools for studying HEV infection, and the amplification of HEV RNA by reverse transcription (RT) followed by the PCR of the cDNA is the most sensitive technique to screen clinical specimens during the course of the disease (4). We show that large-scale identification of HEV in fecal specimens is possible by using a simple modified viral RNA purification method which preserves the integrity of nucleic acids and eliminates potentially inhibitory factors prior to RT-PCR. Therefore, RT-PCR appears to be a useful method for diagnosing and monitoring HEV.

We studied stools and sera collected from patients with elevated levels of transaminase and jaundice who were hospitalized during an hepatitis E outbreak and on the occasion of 10 sporadic non-A, non-B, non-C hepatitis cases in Africa. These patients had elevated levels of alanine aminotransferase and aspartate aminotransferase at the time of sampling. Two *Macaca fascicularis* monkeys (Cy 967 and Cy 479) were also inoculated intravenously with a stool suspension containing HEV (reference strain SAR-55, kindly provided by R. H. Purcell, Bethesda, Md.). Monkey Cy 479 had been inoculated 6 months earlier with the SAR-55 strain and had developed an anti-HEV response. Blood samples and fecal specimens were collected before inoculation and twice weekly after inoculation. The course of HEV infection in the monkeys was monitored by assessing the levels of alanine aminotransferase and aspartate aminotransferase in serum, by detecting HEV RNA in stools and sera, and by determining the anti-HEV antibody kinetics. Suspensions of fecal specimens from humans and monkeys were prepared by vortexing 1 g of feces with 10 ml of phosphate-buffered saline. The suspensions were clarified at 5,000 × *g* for 30 min at 4°C, and aliquots were stored at −70°C. Anti-HEV immunoglobulin G (IgG) in the sera was detected by using a commercial enzyme-linked immunosorbent assay kit (HEV enzyme immunoassay; Abbott Laboratories, North Chicago, Ill.) according to the manufacturer's guidelines. Assays for the detection of Anti-HEV IgM in the available human sera were performed by Abbott Laboratories (Saint Rémy sur Havre, France). HEV RNA was extracted from the fecal suspensions and from serum samples by a modified proteinase K (PK) method and with a commercial kit (RNAzol; Bioprobe Systems, Montreuil sous Bois, France) based on the guanidinium isothiocyanate (GIT) method (2). Briefly, 100 μl of serum or fecal suspension was mixed with 300 μl of lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5% sodium dodecyl sulfate) containing PK (final concentration, 100 μg/ml) and incubated for 60 min at 55°C. Nucleic acids were extracted twice with phenol-chloroform (vol/vol), precipitated with ethanol at −20°C overnight, washed with 75% ethanol, and dissolved in 20 μl of diethylpyrocarbonate-treated water. For the GIT method, 100 μl of serum or fecal suspension was mixed with 100 μl of RNAzol and 20 μl of chloroform. After centrifugation (12,000 × *g*, 5 min), isopropanol precipitation of RNA was carried out at 4°C for 15 min. The RNA precipitate was treated as described above. Sense and antisense synthetic oligonucleotide primers corresponding to the nucleotide sequence of the putative HEV RNA polymerase gene were used for RT-PCR (6). The RT step was carried out in a 20-μl reaction mixture volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.5 mM (each) deoxynucleoside triphosphates (Pharmacia LKB, Uppsala, Sweden), 50 pmol of the sense and antisense outer primers, 40 U of RNase inhibitor (Boehringer, Mannheim, Germany), 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Gaithersburg, Md.), and 5 μl of heat-denatured RNA extracted by either the PK or GIT method. The mixture was incubated at 37°C for 1 h, heated for 5 to 10 min at 95°C, and placed immediately in an ice bath. The first round of PCR was carried out in a total reaction mixture volume of 100 μl containing 5 μl of cDNA in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μmol of each deoxynucleoside triphosphate, 50 pmol of the outer primers ET-R1 (5′-CAG GGC CCC CAA GTT CTT CT-3′) and ET-F1 (5′-GCT CAT TAT GGA GAG AGT GTG G-3′), and 1.25 U of *Taq* DNA polymerase (Pharmacia LKB). Thirty-five cycles of PCR (94°C for 1 min, 52°C for 2 min, and 72°C for 2 min) were carried out. For the second round of PCR, 10 μl

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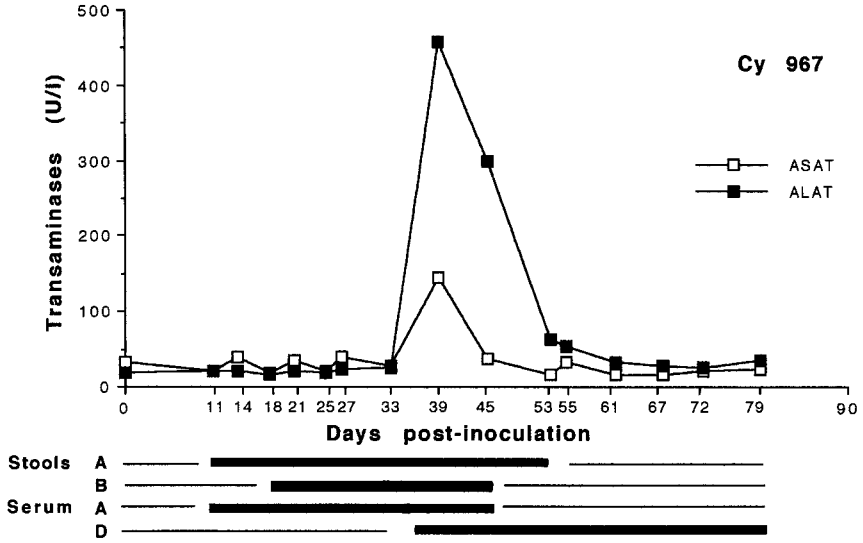

FIG. 1. Biochemical, serological, and virological profiles of HEV infection in monkey Cy 967. At the bottom, the HEV-positive results by PCR (▬) for stool and serum specimens after PK digestion and phenol-chloroform extraction (A) and for stool specimens after GIT extraction (B) are shown. (D) The presence of anti-HEV antibodies (▬) in serum is indicated. ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase.

of the first PCR product was amplified as described above, except that the primers used were internal sense and antisense ET-R2 (5′-TTC AAC TTC AAG ACC ACA GCC-3′) and ET-F2 (5′-GCG TGG ATC TCT GCA GGC C-3′).

To monitor possible contaminations, blood and fecal specimens collected before inoculation of the monkeys and stool samples from healthy humans were included as negative controls for every test. Plasmid DNA (kindly provided by R. H. Purcell) at a minimal positive concentration was used as a template for a similar amplification, to control the yield of the reaction. Finally, the PCR products were analyzed by electrophoresis in a 2% agarose gel followed by ethidium bromide staining, and the specificity of the band was checked by Southern blotting and hybridization with a ^{32}P-random-priming-labeled HEV plasmid (ECL, Amersham, England).

To assess the HEV genome concentration in the HEV suspension used to inoculate the monkeys, semiquantitative analysis was carried out by comparing the minimal positive dilution of the HEV plasmid to the minimal positive dilution obtained with the HEV RNA purified from the injected inoculum. The end-point dilution titer was 10^4 to 10^5 PCR units per ml by the PK method, whereas the GIT method yielded 10^2 to 10^3 PCR units per ml. The threshold of sensitivity of the PK method was estimated to be 5 to 50 genome copies per ml.

In the Cy 967 monkey, biochemical evidence of hepatitis was documented by at least a fourfold increase in serum alanine

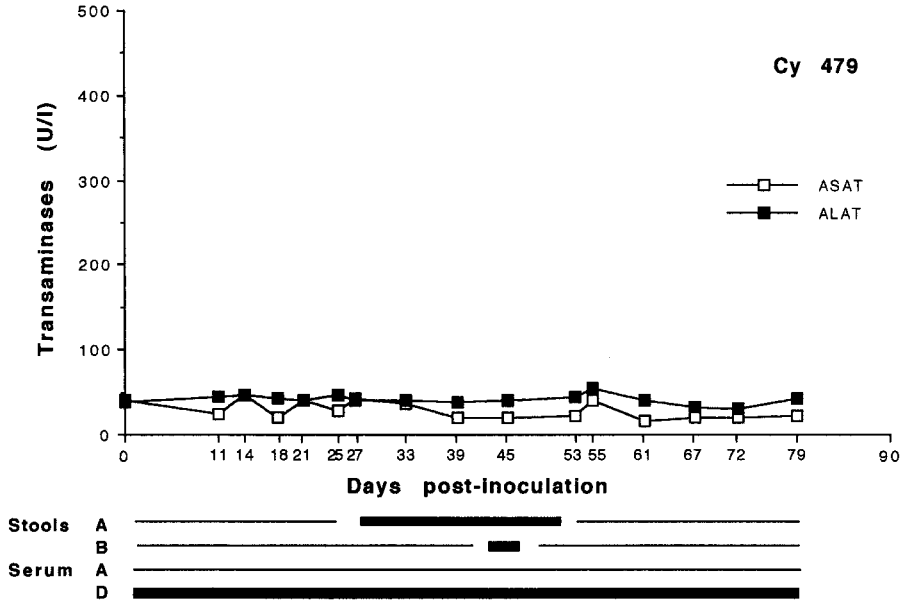

FIG. 2. Biochemical, serological, and virological profiles of HEV infection in monkey Cy 479. Abbreviations and the data at the bottom of the figure are described in the legend to Fig. 1.

TABLE 1. Comparison of the results of two extraction methods for the detection of HEV RNA in feces and sera collected during the acute phase of disease during an HEV outbreak in Africa

Patient no.	Presence of HEV RNA in sample subjected to extraction method indicated					Presence of anti-HEV antibodies	
	Feces			Serum		IgM	IgG
	GIT	PK + electrophoresis[a]	PK + Southern blotting[b]	GIT	PK + electrophoresis[a]		
6	−	+	+	−	+	+	+
12	+	+	+	−	−	ND[c]	+
23	+	+	+	+	+	+	+
25	−	+	+	−	+	−	+
29	−	−	ND	−	−	−	−
36	−	+	+	−	−	+	+
40	−	+	+	−	ND	−	+
56	−	+	+	ND	−	+	+
82	ND	+	+	ND	−	+	+
85	+	+	+	+	+	−	+
86	−	+	ND	−	+	+	+

[a] Analysis was done after 2% gel electrophoresis followed by ethidium bromide staining.
[b] Analysis was done after the sample was blotted onto a nylon membrane and hybridized with ^{32}P-labeled HEV-containing plasmid.
[c] ND, not done.

aminotransferase at 33 to 39 days after inoculation. By the PK method, early detection of HEV RNA in feces and blood was observed on day 11 after inoculation. The duration of viral shedding in feces (4 to 5 weeks) lasted longer than that of viremia in that HEV RNA was still detected in stools 1 week after the end of the viremia. Virus elimination was confirmed at the time that the level of alanine aminotransferase returned to normal and the anti-HEV antibody titer increased (Fig. 1). Monkey Cy 479 had anti-HEV antibodies at the time of challenge, and biochemical analyses did not reveal hepatitis; HEV viremia was never observed. However, by the PK method, HEV genomes were detected in feces from days 27 to 50 whereas by the GIT method HEV RNA was detected only on day 45 (Fig. 2).

In human HEV infection, with the PK method, HEV RNA was detected in the stool samples of 10 of the 11 patients hospitalized for acute hepatitis and in 5 of the 10 corresponding serum samples (Table 1). Hybridization with the ^{32}P-labeled probe confirmed all the positive PCR results. The GIT method was less sensitive than the PK method since among the 10 stool and serum samples tested, only 3 and 2 samples, respectively, were positive. All the patients with stool samples positive for HEV RNA displayed anti-HEV IgG responses, but only six had both IgM and IgG antibodies. In sporadic cases of non-A, non-B, non-C hepatitis, HEV RNA was detected by the PK method in 4 of the 10 stool samples studied, and anti-HEV antibodies were found in all the corresponding serum samples. By the GIT method, no HEV RNA was detected in any stool sample (results not shown).

Our aim was to define a simple method that eliminates substances which could interfere with the sensitivity and specificity of RT-PCR. The detection of RNA viruses in fecal samples is difficult since metabolites and other biological substances can interfere with the reaction (8). Our study demonstrates that the HEV genome appears in the sera and feces of nonimmunized monkeys before the increase in the liver enzymes in sera and remains detectable even after seroconversion. In the monkey challenged with HEV, preexisting anti-HEV IgG prevented HEV viremia, since the HEV genome was not detectable in the serum samples. However, the HEV genome was detected earlier in fecal samples by the PK method, attesting to the sensitivity of this technique. Using the PK method, we were able to detect small amounts of HEV RNA in human stool and serum samples collected during the acute phase of the disease. IgG anti-HEV antibodies were detected but IgM anti-HEV antibodies were not always present in the serum samples. Therefore, to distinguish between present and past infection, HEV viremia or viral fecal shedding must be detected.

The GIT method seems to lyse viral particles less efficiently than the PK method or is unable to eliminate potentially inhibitory factors, leading to false-negative results when amplification of cDNA is carried out under the same conditions. Several matrices have been employed to capture nucleic acids by absorbing RNA or DNA from clinical specimens such as blood and urine (1). The use of glass powder, silicon dioxide, and polyethylene glycol (8000) to improve the amounts of HEV RNA extracted from sera and from stools has been described previously (5, 7). However, these methods are time-consuming and may lead to the loss of some HEV RNA during the purification steps. Moreover, the additional pipetting or handling required may increase the risk of contamination.

In conclusion, for HEV RNA extraction from stool and serum samples, the use of PK digestion and phenol-chloroform extraction prior to RT-PCR gives sensitive and specific results in the screening of clinical specimens.

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